Z-335, a Thromboxane A\textsubscript{2} Receptor Antagonist, Suppresses the Progression of Arachidonic Acid-Induced Hind Limb Gangrene in Rats

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We have developed a new rat model of gangrenous peripheral vascular disease with vascular injury and occlusive thrombi. Rat hind limb gangrene was induced by injecting arachidonic acid (2 mg/kg) into the femoral artery. Using this model, we evaluated the effect of a thromboxane A\textsubscript{2} receptor antagonist, Z-335, on the progression of hind limb gangrene. Z-335 (10 mg/kg/d, p.o.) ameliorated arachidonic acid-induced hind limb gangrene. In contrast, daltroban (10 mg/kg/d, p.o.) and cilostazol (100 mg/kg/d, p.o.) tended to improve the hind limb gangrene but their effects failed to reach statistical significance. Z-335 (10 mg/kg, p.o.) inhibited U-46619-induced, but not collagen-induced, platelet aggregation in rat whole blood. Daltroban (10 mg/kg, p.o.) showed a tendency to inhibit U-46619-induced platelet aggregation. Cilostazol (100 mg/kg, p.o.) did not inhibit U-46619- or collagen-induced platelet aggregation. Histopathological examination of the injured paws showed that Z-335 (10 mg/kg, p.o.) had partly inhibited the formation of occlusive thrombi. These results indicate that the thromboxane A\textsubscript{2} receptor antagonist Z-335 is effective against arachidonic acid-induced hind limb gangrene in rats. Our experiments suggest that Z-335 may be beneficial in the prevention of gangrenous peripheral vascular disease.

Key words Z-335; gangrene; peripheral vascular disease; arachidonic acid

Peripheral vascular disease of the lower limbs often results from chronic vascular insufficiency with occlusive thrombi, which is characterized by the occurrence of intermittent claudication and ischemic gangrene.\textsuperscript{1–3} We have recently developed a rat model of peripheral vascular disease by injecting arachidonic acid into the femoral artery.\textsuperscript{4} This model is characterized by the occurrence of hind limb gangrene with both vascular injury and occlusive thrombi. In addition, we demonstrated that injecting arachidonic acid into the femoral artery enhances markedly the platelet response to a thromboxane A\textsubscript{2} analogue, U-46619, and that the depletion of circulating platelets due to thrombocytopenia suppresses arachidonic acid-induced hind limb gangrene.\textsuperscript{4–6} We have further shown that the combined administration of aspirin and ticlopidine ameliorates hind limb gangrene in this model.\textsuperscript{7} Thus, one of the characteristics of the model is that platelets are involved in the progression of hind limb gangrene.

Thromboxane A\textsubscript{2} released from activated platelets evokes platelet aggregation and vasoconstriction, which may worsen local ischemia in the injured paw.\textsuperscript{7} Although a high dose of aspirin (100 mg/kg, p.o.), a cyclooxygenase inhibitor, is ineffective against arachidonic acid-induced hind limb gangrene,\textsuperscript{7} the failure of this drug may be due to inhibition of prostaglandin I\textsubscript{2} (a vasodilatory and antiplatelet prostanooid) formation. Therefore, it is of interest to examine whether thromboxane A\textsubscript{2} receptor blockade ameliorates arachidonic acid-induced hind limb gangrene.

Z-335 is a potent and orally active thromboxane A\textsubscript{2} receptor antagonist that ameliorates experimental thrombosis.\textsuperscript{4–8} In this study, we tested the effect of Z-335 on arachidonic acid-induced hind limb gangrene in rats. We also examined the effect of cilostazol, a widely used peripheral vascular disease-ameliorating drug.\textsuperscript{9}

MATERIALS AND METHODS

Animals Male Sprague-Dawley rats (8 weeks old, Charles River Japan Inc., Japan) were housed in an air-conditioned room for at least 5 d before we began the present experiments.

Drugs Z-335 ((±)-sodium[2-(4-chlorophenylsulfonylaminomethyl)indan-5-yl]acetate monohydrate),\textsuperscript{9,10} daltroban (4-(2-(4-chlorobenzensulfonylamide)-ethyl)-benzene-acetic acid)\textsuperscript{10} and cilostazol (6-(4-(1-cyclohexyl-1'H-tetrazol-5-yl)-butoxy)-3,4-dihydro-2(1'H)-quinoxaline)\textsuperscript{10} were synthesized in the central research laboratories of Zeria Pharmaceutical Co., Ltd. Sodium arachidonate was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), U-46619, a thromboxane A\textsubscript{2} receptor analogue, was from Funakoshi Co. (Tokyo, Japan) and collagen (Collagenreagent Horm)\textsuperscript{10} was from Nycoomed Co. (Munich, Germany). Z-335, daltroban and cilostazol were suspended in 0.5% (v/v) methyl cellulose solution. Arachidonic acid was dissolved in physiological saline. U-46619 was dissolved in 99.5% (v/v) ethanol and diluted for use with physiological saline.

Arachidonic Acid-Induced Hind Limb Gangrene The procedure was performed according to our previous report.\textsuperscript{4} Rats were fasted for 24 h. Under sodium pentobarbital (40 mg/kg, i.p.) anesthesia the right femoral artery was exposed, and sodium arachidonate (100 µl, 20 mg/ml in saline) was injected into the artery. Test drugs were given orally 1 h prior to arachidonic acid injection and then administered orally for 9 consecutive days. The severity of hind limb gangrene was estimated 10 d after arachidonic acid injection and graded on a scale from 0 to 7: 0, normal; 1, gangrene limited to the claws; 2, gangrene limited to the toes; 3, gangrene extended to the sole; 4, gangrene extended to less than half of the instep; 5, gangrene extended to less than two-thirds of the instep; 6, gangrene extended to more than two-thirds of the instep; 7, gangrene extended to the leg.

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Platelet Aggregation in Whole Blood ex Vivo  Platelet aggregation was measured with a whole-blood aggregometer (model 560, Chrono-Log, U.S.A.). Briefly, the test drug was given orally to rats 24 h prior to blood collection. The rat was anesthetized with ether and blood was drawn from the abdominal aorta into a syringe containing heparin (10 U/ml), immediately after which 500 μl blood was added to 490 μl physiological saline in a cuvette maintained at 37 °C. Then U-46619 (500 nm final concentration) or collagen (750 ng/ml final concentration) was added to the blood sample. The results are expressed as the maximum impedance (Ω) between the 2 electrodes 5 min after adding U-46619 or collagen.

Histopathological Examination of the Injured Paw Z-335 was given orally 1 h prior to arachidonic acid challenge. About 24 h after this challenge, the injured hind limb was fixed with 10% (v/v) formaline, decalcified and embedded in paraffin. Sections through various parts of the paw were stained with hematoxylin and eosin for light microscopic study.

Statistical Analysis Results are expressed as means ± S.E.M. Differences among the groups in the degree of hind limb gangrene were compared using the Mann-Whitney test. Dunnett’s test was used to determine the significance of differences in platelet aggregation among the groups. Differences at p<0.05 were considered significant.

RESULTS

Effect of Z-335 on the Progression of Hind Limb Gangrene Injecting arachidonic acid into the rat femoral artery caused hind limb gangrene. Z-335 (10 mg/kg/d, p.o.) had ameliorated the hind limb gangrene after 10 d. Daltroban (10 mg/kg/d, p.o.) and cilostazol (100 mg/kg/d, p.o.) tended to ameliorate arachidonic acid-induced hind limb gangrene but their effects had not reached statistical significance 10 d after arachidonic acid injection (Fig. 1).

Effect of Z-335 on Platelet Aggregation in Whole Blood Z-335 (10 mg/kg, p.o.) significantly inhibited U-46619-induced platelet aggregation in rat whole blood and daltroban (10 mg/kg, p.o.) tended to inhibit U-46619-induced platelet aggregation, however these drugs did not inhibit collagen-induced platelet aggregation. Cilostazol (100 mg/kg, p.o.) inhibited neither U-46619- nor collagen-induced platelet aggregation (Fig. 2).

Histopathological Examination of the Injured Paw Arachidonic acid injection caused occlusive thrombi and marked denudation of the endothelium or degeneration of the media in the paw arteries. Z-335 (10 mg/kg, p.o.) partly inhibited the formation of occlusive thrombi but failed to prevent vascular injury, including denudation of the endothelium and degeneration of the media (Fig. 3).

DISCUSSION

Injecting arachidonic acid into the rat femoral artery...
causes severe hind limb gangrene, due to occlusive thrombi formation following vascular injury and platelet activation.\textsuperscript{4} Vascular injury and thrombus formation immediately after arachidonic acid challenge will play a partial role in the progression of arachidonic acid-induced hind limb gangrene.\textsuperscript{4} In the expectation of inhibiting the vascular responses, therefore, test drugs were also given before arachidonic acid injection.

In the present study, we chose daltroban, a selective thromboxane A\textsubscript{2} receptor antagonist, and cilostazol, a widely used peripheral vascular disease-ameliorating drug, to evaluate the therapeutic utility of Z-335 as a novel thromboxane A\textsubscript{2} receptor antagonist that ameliorates peripheral vascular disease. The dose of Z-335 that inhibited U-46619-induced platelet aggregation in rat whole blood (10 mg/kg, p.o.), ameliorated arachidonic acid-induced hind limb gangrene. Daltroban (10 mg/kg, p.o.), a selective thromboxane A\textsubscript{2} receptor antagonist, tended to ameliorate hind limb gangrene in this model and to inhibit U-46619-induced platelet aggregation in rat whole blood. Daltroban has been found to possess high selectivity and potency for thromboxane A\textsubscript{2} receptors in vitro\textsuperscript{16,17} and to reduce U-46619-induced lethality in vivo.\textsuperscript{12} However, we have previously shown that, in several experimental thrombosis models, the antithrombotic activity of this compound is weaker than that of Z-335.\textsuperscript{5,6} Therefore, the difference in the ameliorating effects of Z-335 and daltroban in our model seems to be due to the difference in their antithrombotic potencies. In addition, the superior bioavailability of Z-335 in rats may have contributed to the ameliorating effect of this compound in our model (unpublished data). Thus thromboxane A\textsubscript{2} receptor-mediated platelet activation seems to be involved in the progression of hind limb gangrene in our model.

About 24 h after arachidonic acid injection, arterial occlusive thrombi form in the injured paw, which is an important event in the progression of hind limb gangrene.\textsuperscript{4} Accordingly, we performed histopathological examinations of arachidonic acid-injured hind paws to confirm that the antithrombotic activity of Z-335 occurred via its antplatelet action. The histopathological examinations showed that Z-335 had partly inhibited the formation of occlusive thrombi but had not reduced vascular injury in the treated arteries. Probably Z-335 inhibits platelet activation that is secondary to endothelial injury.

Cilostazol is a widely used phosphodiesterase inhibitor which has antplatelet and vasodilatory actions.\textsuperscript{9} The latest clinical studies have demonstrated that cilostazol improves intermittent claudication in cases of peripheral vascular disease.\textsuperscript{13,14} In the present study, cilostazol (100 mg/kg/d, p.o.) showed a tendency to suppress the progression of arachidonic acid-induced hind limb gangrene, but this drug was ineffective against U-46619- or collagen-induced rat platelet aggregation. The ameliorating effect of cilostazol may be due to its vasodilatory action rather than its antplatelet action.

Z-335 inhibits U-46619-induced vasoconstriction of isolated rat aorta more efficiently than daltroban.\textsuperscript{3} Moreover, recent studies have implicated thromboxane A\textsubscript{2} in the reduction of collateral blood flow following arterial thrombosis.\textsuperscript{15,16} In particular, rat peripheral collateral vessels are sensitive to thromboxane A\textsubscript{2}.\textsuperscript{17} Thus blocking thromboxane A\textsubscript{2} receptor-mediated vasoconstriction may be another mechanism by which Z-335 ameliorates arachidonic acid-induced hind limb gangrene.

In summary, we have shown that the thromboxane A\textsubscript{2} receptor antagonist Z-335 ameliorates arachidonic acid-induced hind limb gangrene in rats. This finding suggests that Z-335 may be beneficial in the prevention of gangrenous peripheral vascular disease.

REFERENCES